BCH 462

Single Radial Immunodiffusion and Immuno-electrophoresis

Immunoassays tests include:

1. Precipitation.
2. Agglutination.
3. Immunofluorescence.
4. Radioimmunoassay (RIA).
5. Enzyme-Linked Immuno sorbent Assay (ELISA)
6. Western Blotting.

These tests involve using either or both types of antibodies:

Monoclonal Antibody	Polyclonal Antibody
Consists of one antibody class/subclass which is selective for a single epitope on the antigen	Contains a mixture of antibodies (mainly IgGs), often recognizing multiple epitopes on the antigen
Because of their specificity, they are less likely to cross-react with other proteins, giving lower background than polyclonal antibodies	May contain non-specific antibodies resulting in background staining
Specificity makes them ideal as the primary antibody in an assay, for detecting antigens in tissue, or for affinity purification of antigens	Useful as secondary antibodies or for immunoprecipitation, as they target multiple epitopes providing a more robust detection

Precipitation Reactions:

-This describes the reaction between soluble antibody and soluble antigen in which an insoluble product results, the precipitate.

-These reactions depend on the formation of lattices (cross-links) when antigen and antibody exist in optimal proportions. [it is known as zone of equivalence and appears to us as precipitation].

-Excess of either component reduces lattice formation and subsequent precipitation.

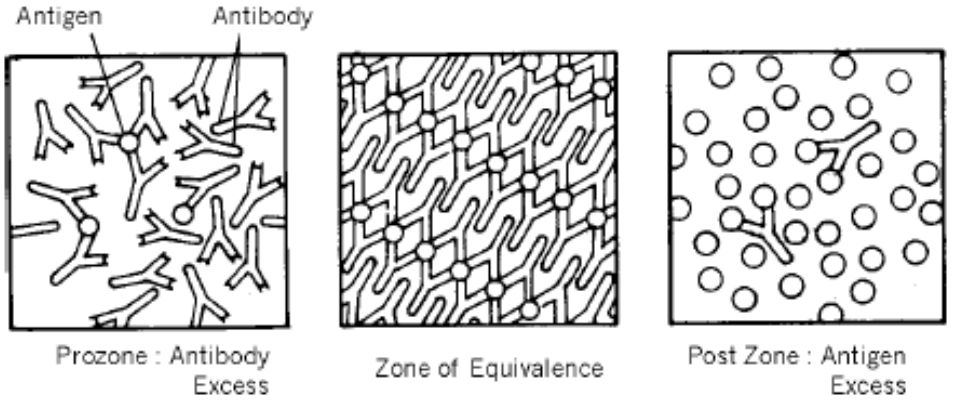
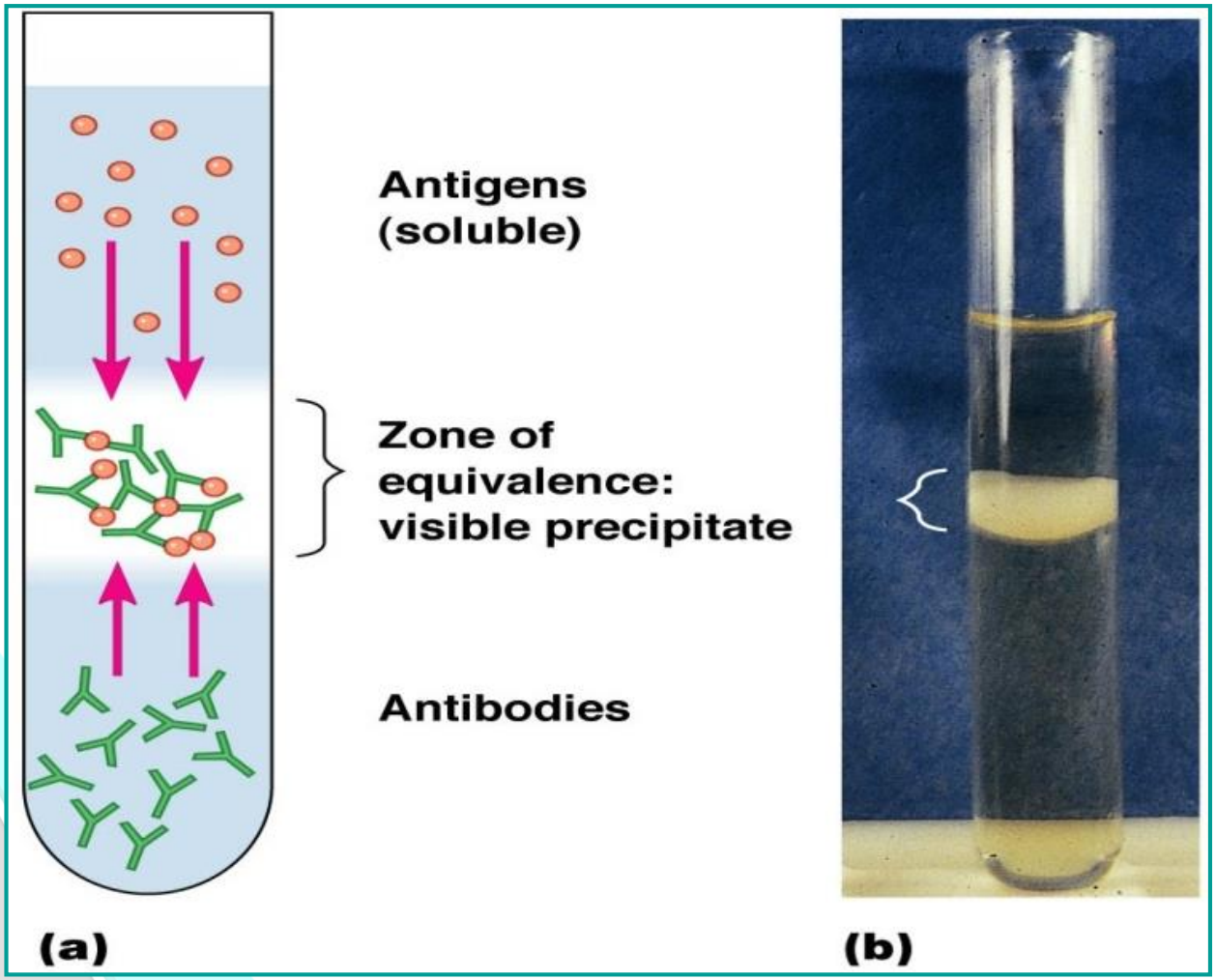


Figure 8.2. The optimal ratio of antigen to antibody (middle) yields an insoluble precipitate in the classic precipitation test. Extreme antibody excess (left) or antigen excess (right), however, does not lead to lattice formation.

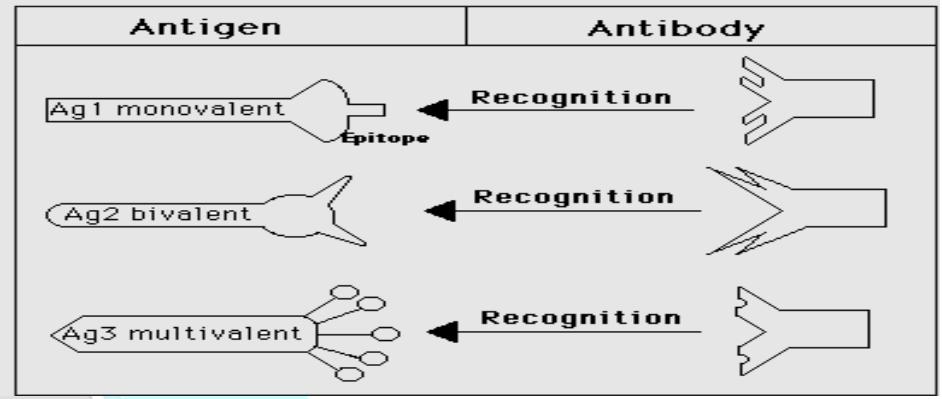

Antigens
(soluble)
Zone of
equivalence:
visible precipitate
Antibodies
(a)
(b)

Precipitation Reactions in general:

Formation of an Ag-Ab lattice depends on the valency of both the antibody and antigen:

-The antibody, must be bivalent; a precipitate will not form with monovalent Fab fragments.

-The antigen, must be either bivalent or polyvalent; that is, it must have at least two copies of the same epitope, or have different epitopes that react with different antibodies present in polyclonal antisera.



Examples of precipitation reaction tests

- Simple Immunodiffusion (ID)

Single Radial ID (RID) (Mancini)

- Electro-Immnodiffusion

Immunoelectrophoresis (IEP)

What is Immunodiffusion technique:

Any technique involving diffusion of antigen or antibody through a semi-solid medium, usually agar or agarose gel, resulting in a precipitin reaction.

Single Radial Immunodiffusion

What is Single Radial Immunodiffusion assay" Mancini method"?

-Is an immunodiffusion technique, used in immunology to determine the quantity "concentration" of an antigen.

-It is suitable for routine use in the diagnostic laboratories.

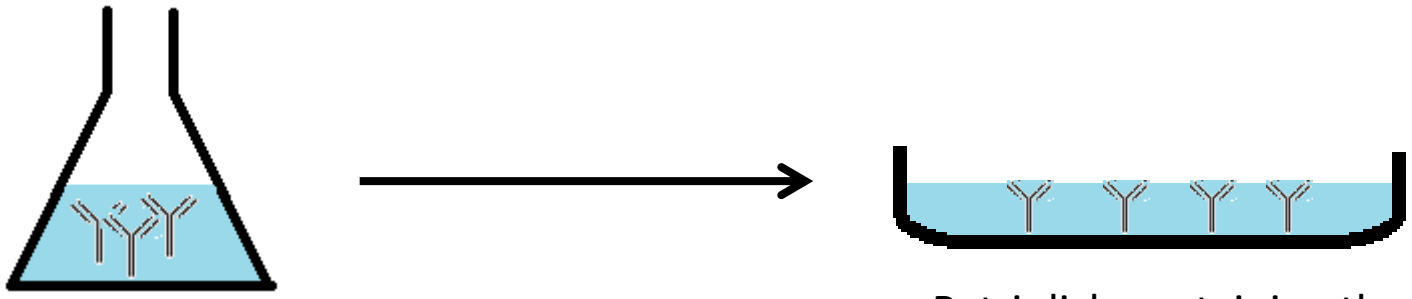
Principle:

-As the antigen diffuses into the medium ' agar or agrose gel' [which containing the fixed antibody], reacts with the antibody, and forms insoluble precipitin complexes, when the antigen and antibody reach the zone of equivalence.

-The quantity of an antigen is determined by measuring the diameters of circles of precipitin complexes surrounding samples of the antigen that mark the boundary between the antigen and an antibody fixed in a medium.

Determination of antigen concentration using radial immunodif

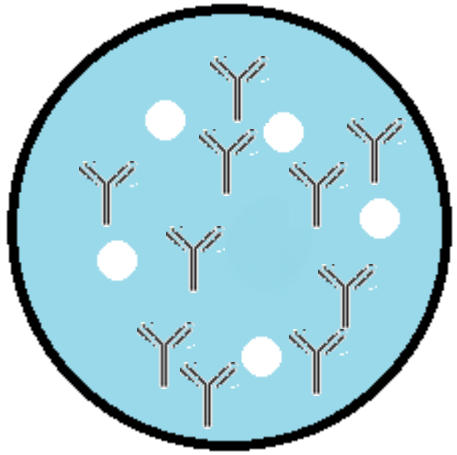
1. Incorporate of antiserum “antibodies” specific for the antigen of interest, into molten agarose. Then pour the mixture in Petri dish.

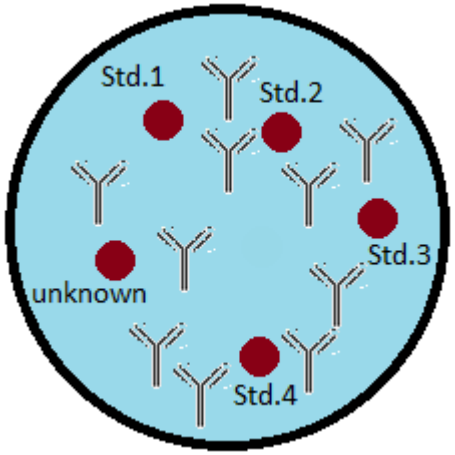


Agarose + antiserum [antibody]

Petri dish containing the uniformly distributed antiserum in the agarose gel.

2- Cut the wells using a gel puncher. Then apply your standard antigen and sample antigen to the wells.





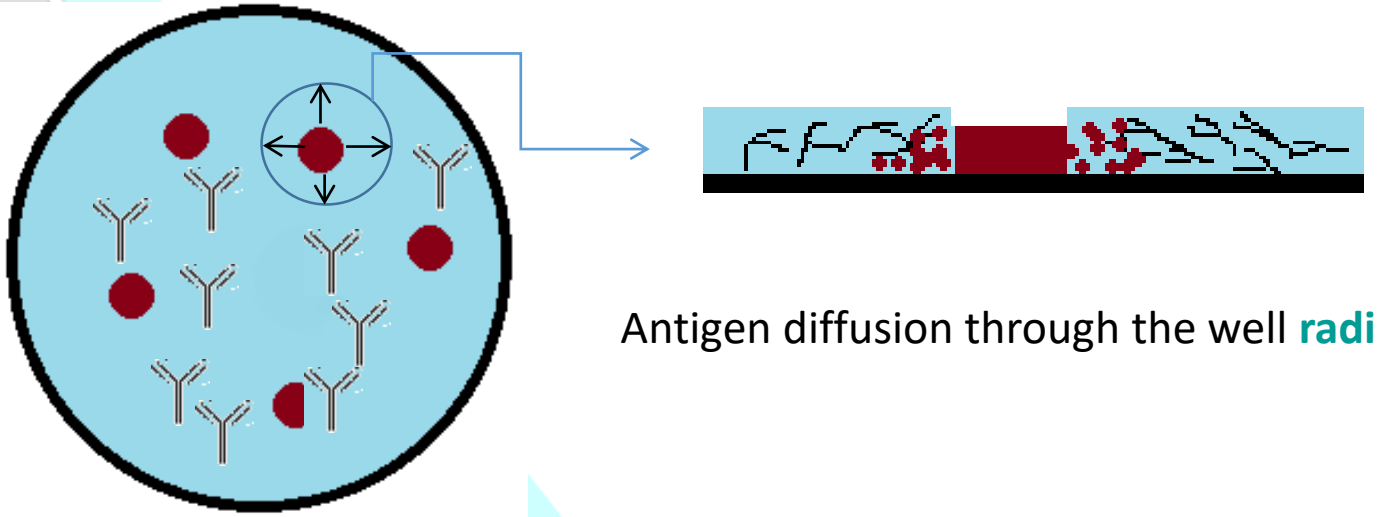
After the antigen is added:

- Initially, as the antigen diffuses through the well, its concentration is relatively high [So, no precipitation will occur].

-However, as antigen diffuses farther and farther from the well, its concentration decrease.

-when its concentration becomes equivalent to that of the antibody fixed in the gel, A ring of antigen-antibody precipitate [precipitin] is formed. **[zone of equivalence]**

-The greater the initial concentration of antigen in the well, the greater the diameter of the precipitin ring.



Antigen diffusion through the well **radially.**

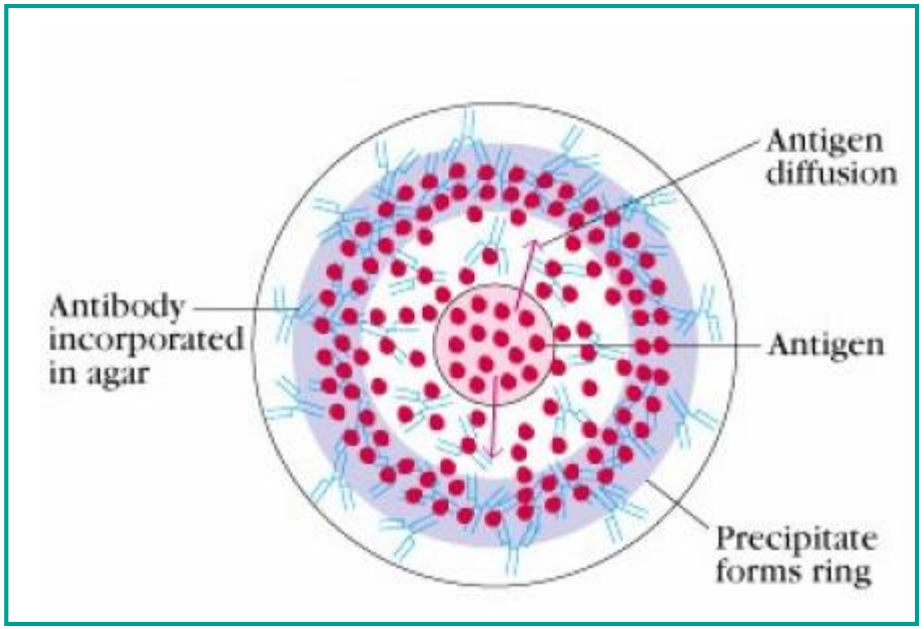


Figure: Radial immunodiffusion test, antigen is filled in the well, antigen start diffusing radially in the gel that contain specific antibody.

3. After 24 to 48 hour, the diameter of circular precipitates formed around the wells then they are measured.

-By measuring the diameters of the precipitin rings formed by known antigen concentrations "standard antigens concentrations", a standard curve can be constructed.

-The unknown antigen concentration, can then be found by simple interpolation having measured the diameters of the respective precipitin rings.

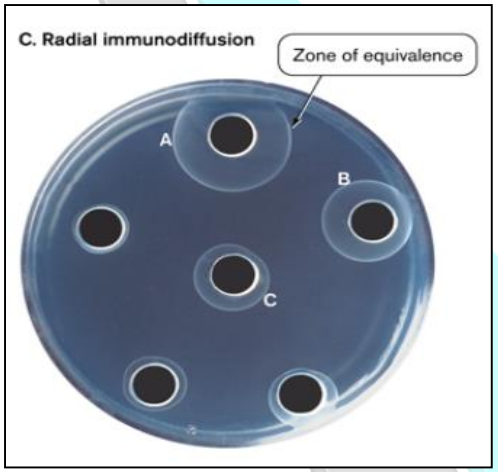


Figure: The precipitin ring is formed around the wells of the samples.

Note that: the diameters of precipitin rings are directly proportional to antigen concentration.

4. A plot of precipitation ring diameters versus concentrations is made for the samples with known antigen concentrations.

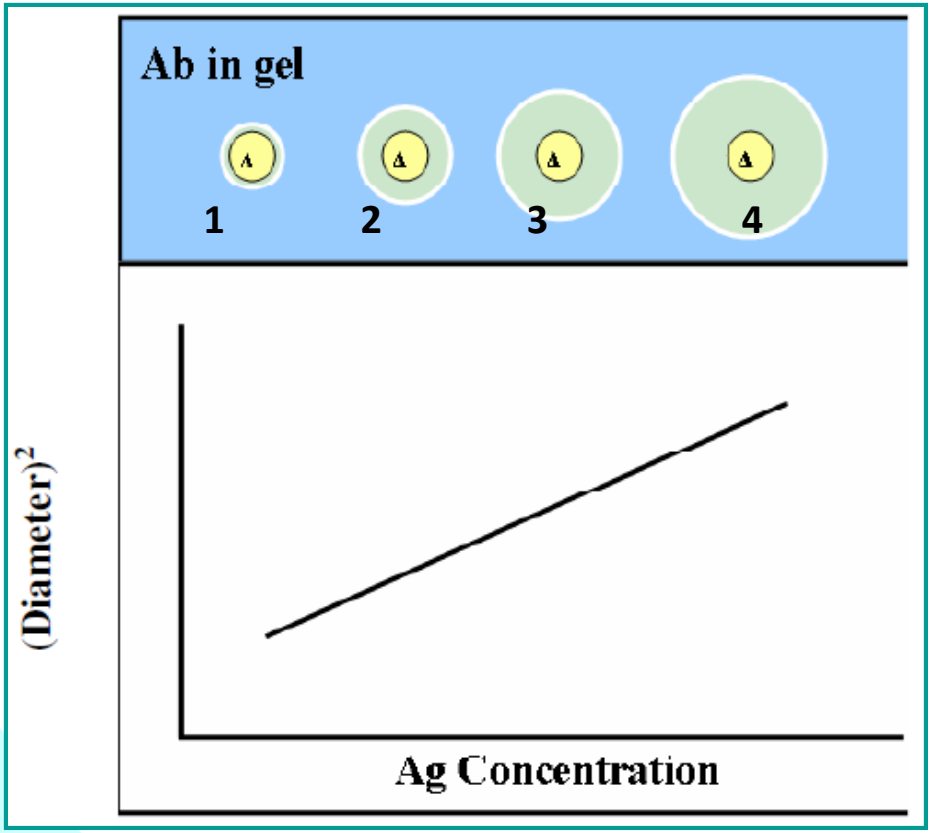
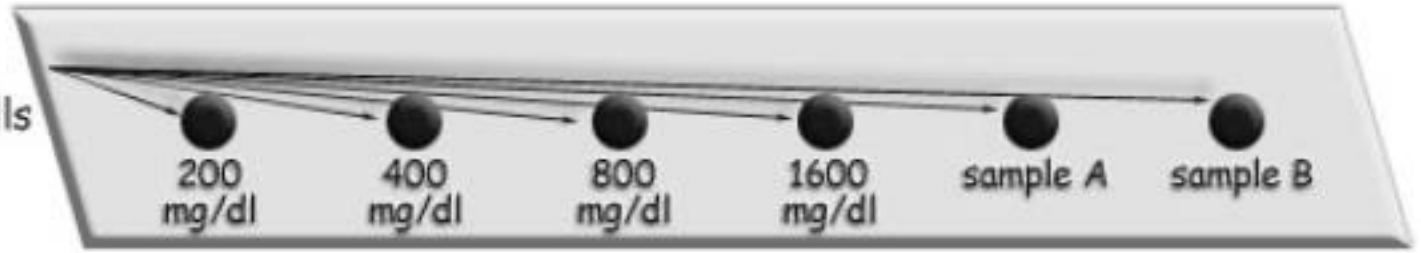


Figure: wells (1-4), contain known standard concentrations of protein under study ”antigen”.

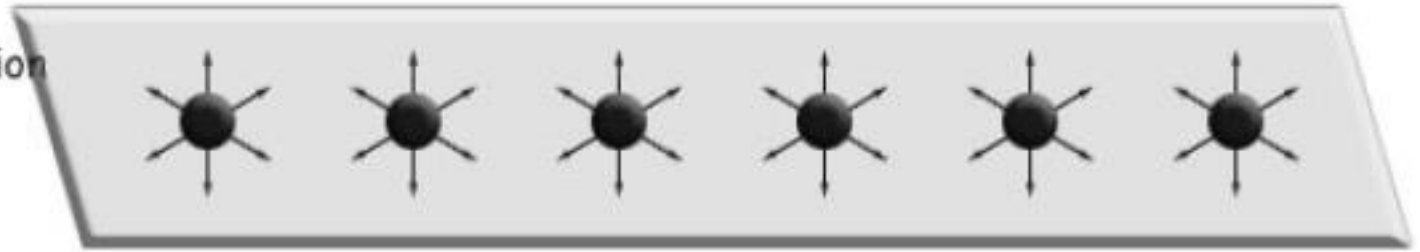
The graph shows the resulting calibration curve from which, an unknown concentration of the antigen can be determent.

REAGENTS:
agar gel impregnated with antiglobulins (anti-IgG)

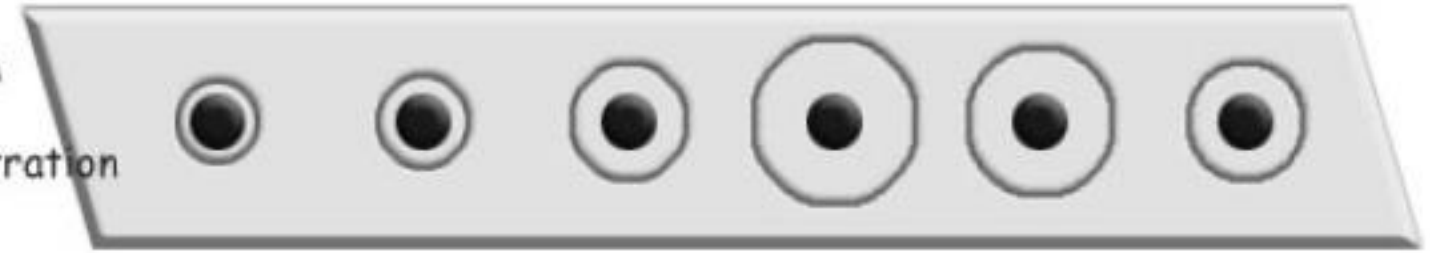
1. add serum sample & IgG standards to wells



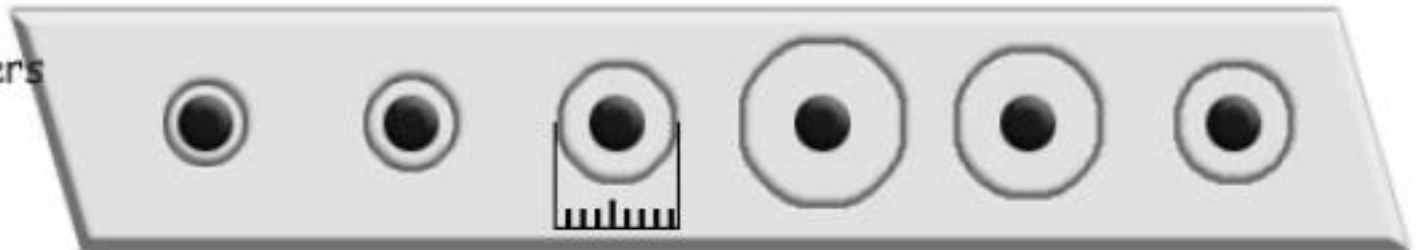
2. allow time for diffusion of IgG's into gel



3. precipitin rings form at site of optimal IgG:anti-IgG concentration



4. measure ring diameters (proportional to IgG concentrations)



Immuno-electrophoresis

Immuno-electrophoresis:

-Technique based on the principles of electrophoresis of antigens and immunodiffusion of the electrophoresed antigens with a specific antiserum to form precipitin bands.

-It is used in clinical laboratories to detect the presence or absence of proteins in the serum.

-This technique is useful in determining whether a patient produces abnormally low amounts of one or more isotypes of Ig , characteristic of certain immunodeficiency diseases.

-It can also show whether a patient overproduces some serum protein, such as albumin, immunoglobulin, or transferrin.

During:

Electrophoresis, molecules placed in an electric field acquire a charge and move towards appropriate electrode. Mobility of the molecule is dependent on a number of factors:

-Size of molecules to be separated.

-Concentration of agarose gel.

-Voltage applied.

-The buffer used for electrophoresis.

Immunodiffusion:

Antigens resolved by electrophoresis are subjected to immunodiffusion with antiserum added in a trough cut in the agarose gel. Antigen-antibody complex precipitates at the zone of equivalence to form an opaque arc shaped line in the gel.

Principle:

A gel is prepared with a well to add the antigen in it.

1. The antigen mixture, is first electrophoresed to separate its components by charge.

2. Troughs are then cut into the agar gel parallel to the direction of the electric field.

3. Antiserum is added to the troughs.

4. Antibody and antigen then diffuse toward each other.

5. Lines of precipitation [arcs], will be produced where they meet in appropriate proportions [at the zone of equivalence].

6. The precipitin line indicate the presence of the antigen- antibody complex. While the absence of precipitin line indicates the absence of antigen- antibody complex.

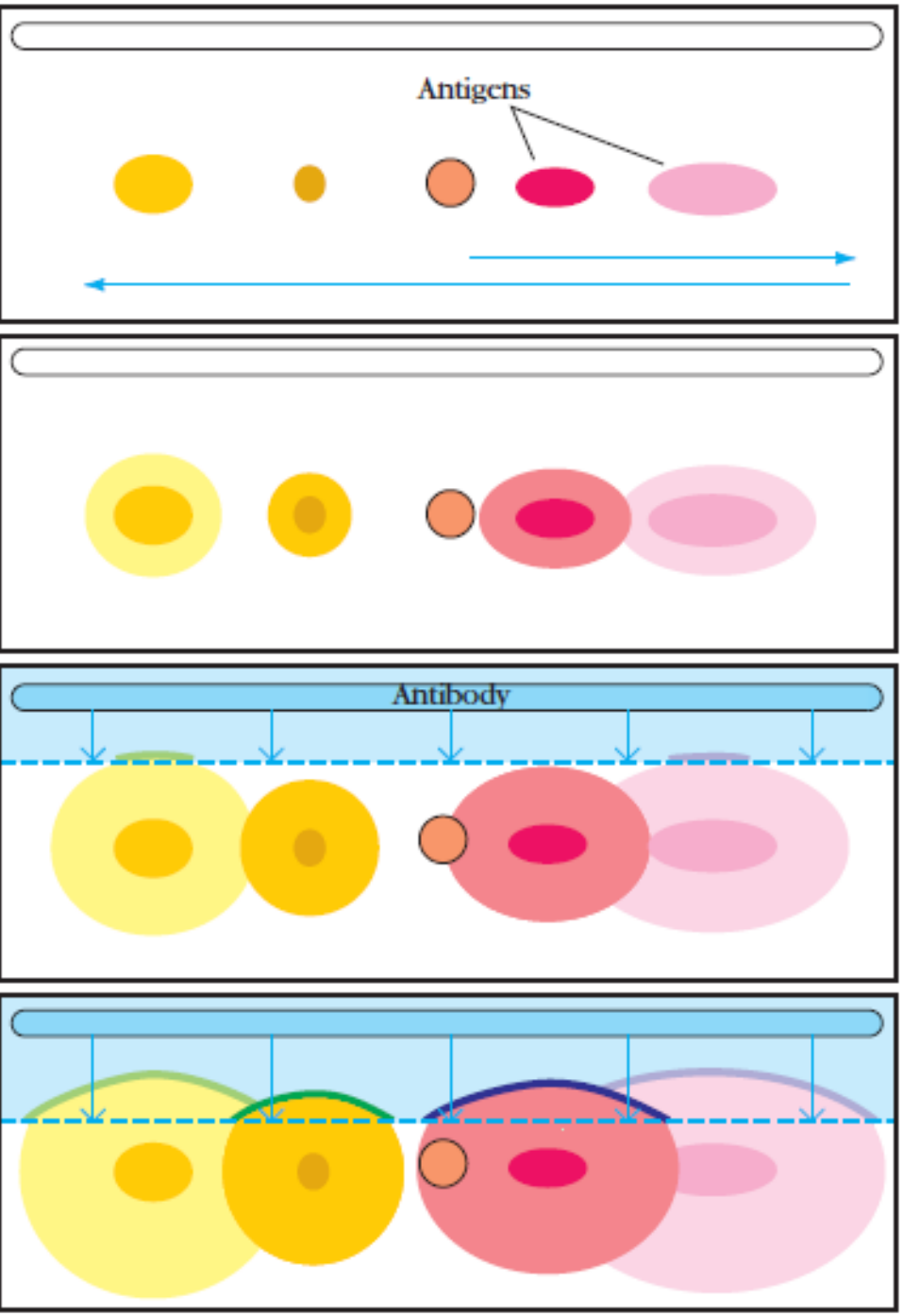


Figure:
Immunoelectrophoresis of an antigen mixture.

-An **antigen** preparation **(orange)** is first electrophoresed, which separates the component antigens on the basis of charge.

-**Antiserum (blue),** is then added to troughs on one or both sides of the separated antigens and allowed to diffuse.

-In time, lines of precipitation (colored arcs) form where specific antibody and antigen interact.

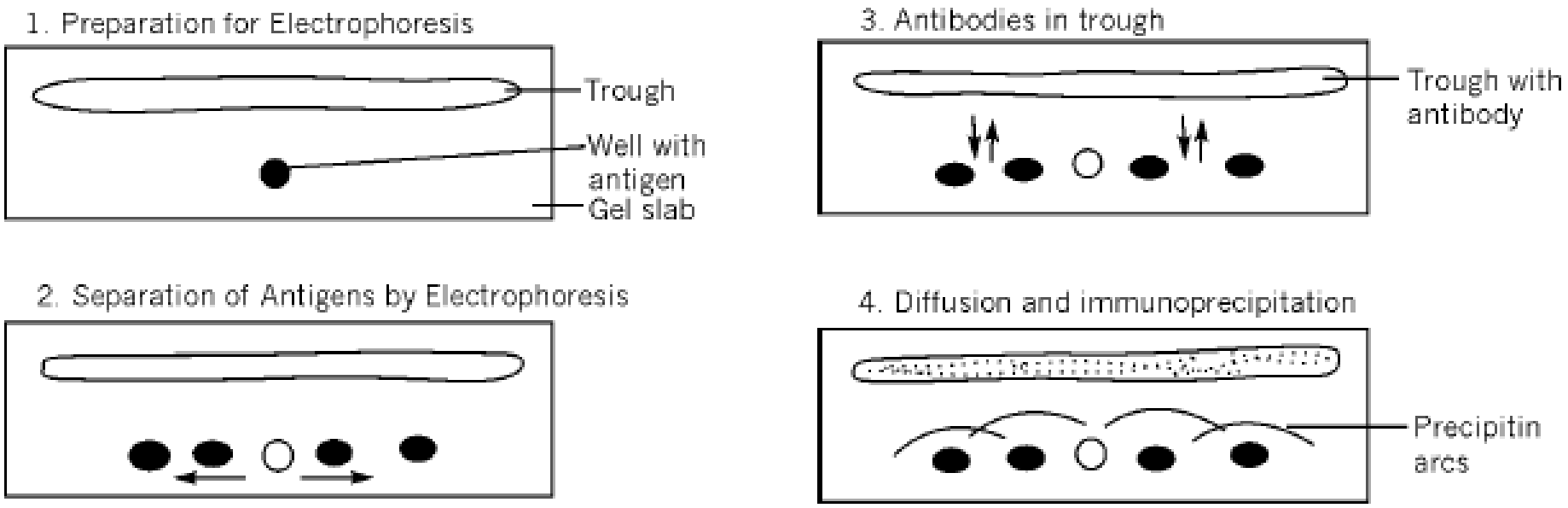
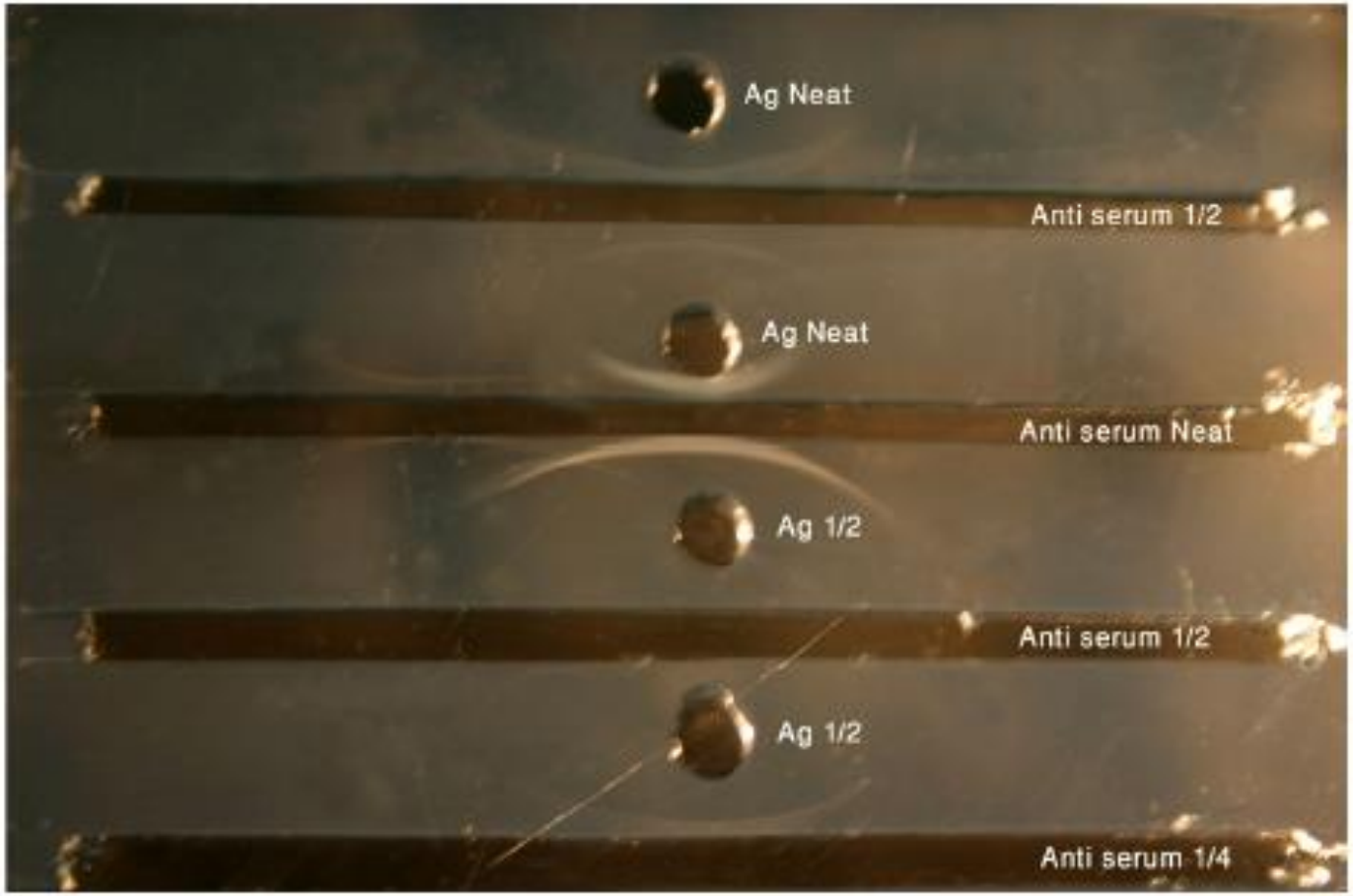


Figure 8.8. Technique of immunoelectrophoresis : (1) Agar gel slab is prepared, a trough and well are cut and the well is filled with antigen. (2) Antigens are separated by electrophoresis and the pH of the gel is chosen so that positively charged proteins move to the negative electrode and negatively charged proteins move to the positive electrode. (3) After antigen separation, the trough is filled with antiserum which is left to diffuse. (4) The separated antigens and the antibodies in the trough diffuse, interact and form precipitin arcs. Immunoelectrophoresis permits the comparison of complicated mixtures of antigens found in human serum.


Ag Neat
Anti serum 1/2
Ag Neat
Anti serum Neat
Ag 1/2
Anti serum 1/2
Ag 1/2
Anti serum 1/4